

replaced by 13 from the vector plus two from the PCR primer (Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg-Ile-Asn-Ser) (SEQ ID NO:29).

Our goal was to generate enzymes that lacked the ability to synthesize DNA, but retained the ability to cleave nucleic acids with a 5' nuclease activity. The act of primed, templated synthesis of DNA is actually a coordinated series of events, so it is possible to disable DNA synthesis by disrupting one event while not affecting the others. These steps include, but are not limited to, primer recognition and binding, dNTP binding and catalysis of the inter-nucleotide phosphodiester bond. Some of the amino acids in the polymerization domain of DNAPEcI have been linked to these functions, but the precise mechanisms are as yet poorly defined.

One way of destroying the polymerizing ability of a DNA polymerase is to delete all or part of the gene segment that encodes that domain for the protein, or to otherwise render the gene incapable of making a complete polymerization domain. Individual mutant enzymes may differ from each other in stability and solubility both inside and outside cells. For instance, in contrast to the 5' nuclease domain of DNAPEcI, which can be released in an active form from the polymerization domain by gentle proteolysis [Setlow and Kornberg, *J. Biol. Chem.* 247:232 (1972)], the *Thermus* nuclease domain, when treated similarly, becomes less soluble and the cleavage activity is often lost.

Using the mutant gene shown in Fig. 4B as starting material, several deletion constructs were created. All cloning technologies were standard (Sambrook *et al.*, *supra*) and are summarized briefly, as follows:

Fig. 4C: The mutTaq construct was digested with PstI, which cuts once within the polymerase coding region, as indicated, and cuts immediately downstream of the gene in the multiple cloning site of the vector. After release of the fragment between these two sites, the vector was re-ligated, creating an 894-nucleotide deletion, and bringing into frame a stop codon 40 nucleotides downstream of the junction. The nucleotide sequence of this 5' nuclease (clone 4C) is given in SEQ ID NO:9.

Fig. 4D: The *mutTaq* construct was digested with *NheI*, which cuts once in the gene at position 2047. The resulting four-nucleotide 5' overhanging ends were filled in, as described above, and the blunt ends were re-ligated. The resulting four-nucleotide insertion changes the reading frame and causes termination of translation ten amino acids downstream of the mutation. The nucleotide sequence of this 5' nuclease (clone 4D) is given in SEQ ID NO:10.

Fig. 4E: The entire *mutTaq* gene was cut from pTTQ18 using *EcoRI* and *SalI* and cloned into pET-3c, as described above. This clone was digested with *BstXI* and *XcmI*, at unique sites that are situated as shown in Fig. 4E. The DNA was treated with the Klenow fragment of DNAPEc1 and dNTPs, which resulted in the 3' overhangs of both sites being trimmed to blunt ends. These blunt ends were ligated together, resulting in an out-of-frame deletion of 1540 nucleotides. An in-frame termination codon occurs 18 triplets past the junction site. The nucleotide sequence of this 5' nuclease (clone 4E) is given in SEQ ID NO:11, with the appropriate leader sequence given in SEQ ID NO:30. It is also referred to as Cleavase® BX.

Fig. 4F: The entire *mutTaq* gene was cut from pTTQ18 using *EcoRI* and *SalI* and cloned into pET-3c, as described above. This clone was digested with *BstXI* and *BamHI*, at unique sites that are situated as shown in the diagram. The DNA was treated with the Klenow fragment of DNAPEc1 and dNTPs, which resulted in the 3' overhang of the *BstXI* site being trimmed to a blunt end, while the 5' overhang of the *BamHI* site was filled in to make a blunt end. These ends were ligated together, resulting in an in-frame deletion of 903 nucleotides. The nucleotide sequence of the 5' nuclease (clone 4F) is given in SEQ ID NO:12. It is also referred to as Cleavase® BB.

Fig.4G: This polymerase is a variant of that shown in Figure 4E. It was cloned in the plasmid vector pET-21 (Novagen). The non-bacterial promoter from bacteriophage T7, found in this vector, initiates transcription only by T7 RNA polymerase. See Studier and Moffatt, *supra*. In a suitable strain, such as (DES)pLYS, the gene for this RNA polymerase is carried on the bacterial genome under control of

the *lac* operator. This arrangement has the advantage that expression of the multiple copy gene (on the plasmid) is completely dependent on the expression of T7 RNA polymerase, which is easily suppressed because it is present in a single copy. Because the expression of these mutant genes is under this tightly controlled promoter, potential problems of toxicity of the expressed proteins to the host cells are less of a concern.

The pET-21 vector also features a "His*Tag", a stretch of six consecutive histidine residues that are added on the carboxy terminus of the expressed proteins. The resulting proteins can then be purified in a single step by metal chelation chromatography, using a commercially available (Novagen) column resin with immobilized Ni⁺⁺ ions. The 2.5 ml columns are reusable, and can bind up to 20 mg of the target protein under native or denaturing (guanidine*HCl or urea) conditions.

E. coli (DES)pLYS cells are transformed with the constructs described above using standard transformation techniques, and used to inoculate a standard growth medium (*e.g.*, Luria-Bertani broth). Production of T7 RNA polymerase is induced during log phase growth by addition of IPTG and incubated for a further 12 to 17 hours. Aliquots of culture are removed both before and after induction and the proteins are examined by SDS-PAGE. Staining with Coomassie Blue allows visualization of the foreign proteins if they account for about 3-5% of the cellular protein and do not co-migrate with any of the major protein bands. Proteins that co-migrate with major host protein must be expressed as more than 10% of the total protein to be seen at this stage of analysis.

Some mutant proteins are sequestered by the cells into inclusion bodies. These are granules that form in the cytoplasm when bacteria are made to express high levels of a foreign protein, and they can be purified from a crude lysate, and analyzed by SDS-PAGE to determine their protein content. If the cloned protein is found in the inclusion bodies, it must be released to assay the cleavage and polymerase activities. Different methods of solubilization may be appropriate for different proteins, and a variety of methods are known. *See e.g.*, Builder & Ogez, U.S. Patent No. 4,511,502 (1985); Olson, U.S. Patent No. 4,518,526 (1985); Olson & Pai, U.S. Patent No.